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CDKG1 protein kinase is essential for synapsis and male meiosis at high ambient temperature in *Arabidopsis thaliana*

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The *Arabidopsis* cyclin-dependent kinase G (CDKG) gene defines a clade of cyclin-dependent protein kinases related to CDK10 and CDK11, as well as to the enigmatic Ph1-related kinases that are implicated in controlling homeologous chromosome pairing in wheat. Here we demonstrate that the CDKG1/CYCLINL complex is essential for synapsis and recombination during male meiosis. A transfer-DNA insertional mutation in the *cdkg1* gene leads to a temperature-sensitive failure of meiosis in late Zygotene/Pachytene that is associated with defective formation of the synaptonemal complex, reduced bivalent formation and crossing over, and aneuploid gametes. An aphentotypic insertion in the cyclin L gene, a cognate cyclin for CDKG, strongly enhances the phenotype of *cdkg1-1* mutants, indicating that this *cdk*-cyclin complex is essential for male meiosis. Since CYCLINL, CDKG, and their mammalian homologs have been previously shown to affect mRNA processing, particularly alternative splicing, our observations also suggest a mechanism to explain the widespread phenomenon of thermal sensitivity in male meiosis.

Replication and segregation of DNA underpins the reproduction of all cells. In organisms that undergo sexual reproduction, the nucleus carries two copies of each chromosome (or homologs) that replicate and recognize each other before the meiotic reduction division. This process allows the homologs to pair and exchange genetic material before segregation. A proteinaceous structure, known as the synaptonemal complex (SC), forms along the length of each set of paired chromosomes and subsequently forms physical connections between the homologs. Recombination between the homologs involves first the formation of DNA double-strand breaks (DSBs) that are either repaired as noncrossover or crossover (CO) products. Chiasmata, the cytological manifestation of COs, provide a physical connection between the homologs that persists after the SC is disassembled.

Many of the proteins involved in meiotic recombination and homologous chromosome synapsis have been identified. At the leptotene stage of prophase I, the chromosome axes are elaborated along the conjoined bases of the sister chromatids. Concomitantly, Spo11-dependent DSBs form and are processed at the axes where the recombination machinery is assembled. This enables the alignment of homologous chromosomes and is dependent on the strand-exchange proteins Rad51 and DMC1. During zygotene, the SC starts to form between the homologs through the polymerization of the central element protein ZYP1 which brings the homologous chromosomes into close apposition. SC formation is complete by pachytene, during which the final stages of recombination are complete. The SC breaks down at the end of pachytene, and the chromosomes condense further during diplotene/diakinesis. At metaphase the bivalents held together by chiasmata are arranged at the metaphase plate, and the first meiotic division occurs.

Although many of the structural proteins involved in the progression of meiotic prophase have been identified (reviewed by ref. 1), regulators are still poorly understood. Cyclin-dependent protein kinases (CDKs) regulate diverse cellular functions in both

mitotic and meiotic cell cycles. Cdc28 in budding yeast has a role in Spo11-dependent DSB formation and is necessary for SC assembly (2). CDK2 is required to prevent nonhomologous chromosome pairing in mice (3), and the *Arabidopsis* homolog CDKA;1 is necessary for the meiotic process (4), perhaps through regulation of retinoblastoma-related protein (Rbr) activity (5). *Ph1*, a complex locus containing multiple copies of CDK-like genes (6), enhances the fidelity of chromosome pairing in hexaploid wheat (7), such that homologous chromosomes cross over but homeologs do not (8). The pachytene checkpoint that couples pairing and recombination in animals can be overcome by protein phosphatase inhibitors (9). Ph1 also affects histone phosphorylation and chromatin structure (7), but its precise mechanism remains unclear. Although the *Arabidopsis* genome does not contain a homolog of Ph1 kinases, we previously identified CDKG as the most closely related kinase (10), one which is also conserved across plants and animals. We therefore undertook a detailed study of CDKG function in *Arabidopsis* using a combined genetic and cytological approach and demonstrate that the CDKG/CYCLINL complex is essential for the final steps of chromosome synapsis during male gamete formation.

Results

A transfer-DNA (T-DNA) insertion in *CDKG1* (At5g63370) was verified in the line, SALK_075762 (11), using PCR and sequencing of the resultant PCR product. In homozygous mutant

Significance

Homologous chromosome pairing ensures that each product of meiosis contains a full haploid genome complement and is particularly important in allopolyploid plants which contain multiple, genetically related sets of chromosomes. In domesticated allohexaploid wheat, the *Ph1* locus drives homologous bivalent formation and is required to maintain genome stability and disomic inheritance. In this study, we show that the most closely related gene in *Arabidopsis*, the cyclin-dependent protein kinase CDKG1, also functions in chromosome pairing and is required for the completion of synapsis in male meiocytes at high ambient temperature. This class of CDK has been previously implicated in RNA splicing and may be responsible for the intrinsic temperature sensitivity of meiosis in males.

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Table 1. Reciprocal cross between *cdkg1-1* mutant and WT plants

Parental genotype (Female × Male)	Seed number	Total siliques	Average number per silique
Col0 × Col0	221	10	22.1
<i>cdkg1-1</i> × Col0	243	12	20.2
Col0 × <i>cdkg1-1</i>	5	10	0.5
<i>cdkg1-1</i> × <i>cdkg1-1</i>	6	10	0.6

(Fig. 3A). Three-dimensional reconstruction of nuclei, used to examine bivalent synapsis in more detail [Fig. 3B and Fig. S4 (15)], revealed considerable variation in ZYP1 loading between mutant meiocytes (Fig. S3), with individual chromosome pairs being variably synapsed and partially ZYP1-loaded. In WT, all bivalents were normally synapsed, whereas this was never observed in the mutant nuclei, with bivalents synapsing an average of 29.92% (range 0–73.76%, SD = 0.185, $n = 10$). These observations indicate that the initial loading of ZYP1 (and bivalent recognition) is independent of CDKG1, but that the extended loading of ZYP1 from these initiation sites is CDKG-dependent at high ambient temperature.

To evaluate whether recombination-related processes were also defective in the mutant, we used immunostaining with antibodies against the recombination proteins, DMC1 and RAD51 (16). Both these proteins were recruited normally in WT and mutant and at both temperatures (Fig. 4A), suggesting that the processes associated with DSB formation and early repair are not affected. Mature recombination nodules were detected with α -MLH1 antibody (17, 18) (Fig. 4B). As expected, MLH1 foci were found to always be associated with ZYP1 segments in both mutant and WT nuclei. In WT nuclei grown at 23 °C the average number of MLH1 foci at zygotene/pachytene stages was 9.5 ± 2.1 ($n = 15$), and this number was reduced to 2.5 ± 2.4 ($n = 24$) in the mutant at the same temperature (P value 4.9×10^{-11} , $P < 0.001$). However, at 12 °C there was no significant difference (P value 0.015, $P < 0.001$) between the number of MLH1 foci in the WT (9.9 ± 1.4 , $n = 9$) and the *cdkg1-1* mutant nuclei (7.9 ± 1.8 , $n = 10$), indicating that CDKG1 is required to maintain chiasma frequency at higher temperatures. These data also compare very well with estimates of chiasma frequency at metaphase I in summer-grown greenhouse material using the method described by ref. 19: 3.8 per cell in *cdkg1-1*, compared with 9.7 per cell in WT.

CDKG1–CYCLINL Complex Is Required for Male Fertility. A systematic protein–protein interaction study (20) indicated that that CYCLINL is the cognate cyclin for CDKG. We therefore examined the phenotypes and genetic interactions of insertion mutants in these three genes. Knockout mutations in the *CYCLINL* gene (*mos12-2*) are lethal (21), so we sought alleles with partial loss of function. An allele with a T-DNA insertion in the 3' end of the gene leading to the production of partial *cyclinL* mRNA was identified and confirmed by RT-PCR (Fig. S1) and called *cycL1-1*. *cycL1-1* homozygous lines were fully fertile at both 12 and 23 °C. Pairwise crosses were made between *cdkg1-1* and *cyclinL* lines to produce heterozygous F_1 individuals. Double-homozygous mutant *cycL1-1/cdkg1-1* plants, produced by allowing an F_2 plant with the genotype *cycL1-1/cycL1-1*; *cdkg1-1/+* to self-pollinate and genotyping the progeny, were essentially sterile (fewer than 15 seeds per plant) under all conditions tested (Fig. S5A). Loss of *CYCLINL* in a *cdkg1-1* homozygous background, therefore, strongly enhanced the meiotic phenotype, such that the double-homozygous mutant grown at either temperature resembled the *cdkg1-1* homozygous mutant grown at 23 °C with regard to the cytology of meiosis (Fig. S5B) and pollen formation (Fig. S5C). Loading of ZYP1 at 12 and 23 °C was essentially identical to the single homozygous *cdkg1-1* mutant at 23 °C with small segments of ZYP1 loading, coinciding with limited regions of synapsis and supporting the notion that the CDKG1–CYCLINL complex is required for complete polymerization of ZYP1.

Discussion

The mutual recognition and physical association of homologous chromosomes as structurally connected bivalents during the first reduction division of meiosis is critically important for their accurate segregation. Missegregation defects underlie many genetic diseases in animals and humans. Conversely, the ability to manipulate the stringency of pairing could release novel variation and be an important tool in plant breeding where there is an urgent need to introduce new traits by means of recombination using crosses between phenotypically distinct varieties (22). As potential regulators that can themselves be manipulated, kinases could be attractive tools in breeding (23). Loss of CDKG1–CYCLINL function leads to a failure to consummate the physical association of cognate homologs and has dramatic consequences on the fidelity of chromosome segregation.

CDK–Cyclin Regulation of Meiosis. As with mitosis, reversible phosphorylation of key proteins plays diverse regulatory roles in meiosis. Several CDKs and cyclins have been identified where mutations lead to a variety of specific defects. CDKA (or CDK1 in animals) is central to mitotic progression and is also required for the meiotic program (4). A phosphomimic substitution, T161D, in T-loop of CDKA partially restores the primary vegetative defect of *cdka-1* mutants but is unable to support the meiotic program, indicating that CDKA function is perhaps even more critical during meiosis. At least two different cyclins that interact with CDKA also lead to meiotic defects. *TARDY ASYNCHRONOUS MEIOSIS* encodes CYCLIN A1;2 (CYCA1;2), and *tam* mutants form dyads instead of tetrads (24). Meiosis progresses asynchronously with delays in pachytene of both meiosis I and meiosis II. A null mutant leads to progressive polyploidization and loss of fertility (25). SOLO DANCERS (SDS), a plant-specific cyclin, is required for chromosome pairing and/or synapsis of homologous chromosomes during prophase I, the absence of which leads to greatly reduced levels of meiotic recombination (26). In mammals, the structurally related kinase CDK2 is essential for fertility, blocking meiosis at pachytene with incomplete synapsis (3). This differs radically from the *cdkg1-1* phenotype, however, in that the chromosomes are extensively tangled by nonhomologous synapsis.

Other kinases have been implicated specifically in chromosome pairing in plants. The *Ph1* locus of chromosome 5B of allohexaploid wheat contains (among other elements) a cluster of defective CDK-related genes (23, 27) that suppress the expression of unlinked, but structurally related, CDK genes and CDK activity as measured by Histone H1 phosphorylation (7). The transsilencing of these CDKs by *Ph1* appears to drive homologous bivalent formation by preventing pairing between homeologs from its three constituent chromosome sets. The increased stringency of meiotic chromosome pairing may be mediated via changes in replication and reduced histone H1 phosphorylation, but specific mechanisms were not elaborated. Deletion of *Ph1* increases CDK activity and permits homeologous chromosome pairing, whereas increasing the dosage of *Ph1* (as in lines trisomic for the long arm of Chromosome 5B) suppresses synapsis and chiasma formation even between homologs (28). The activity of CDKG1 in *Arabidopsis* therefore parallels that of *Ph1* in bread wheat, insofar as the mutant has defective pairing that phenocopies the effect of additional copies of the *Ph1* locus in wheat. Given the effect of okadaic acid on the

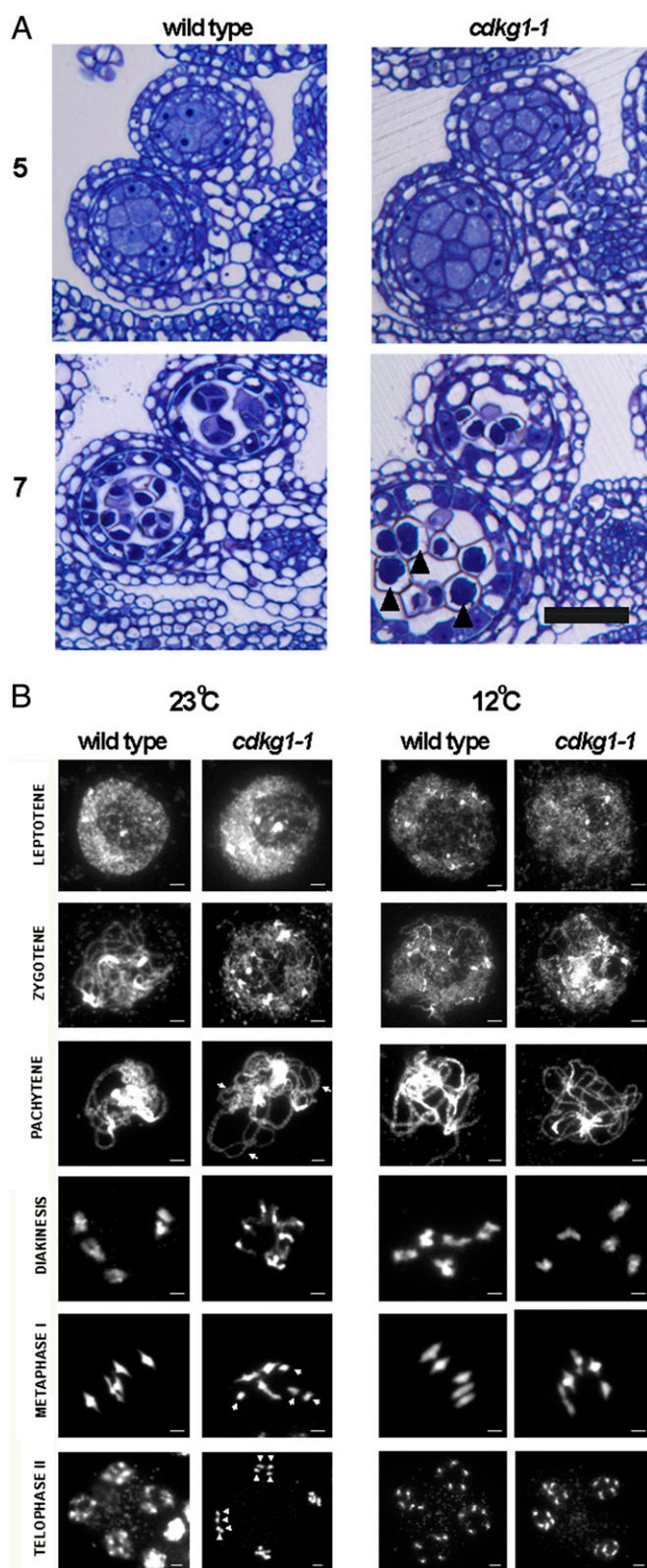


Fig. 2. *cdkg1-1* mutants are conditionally defective in meiosis. (A) Resin sections of staged (5, 7) anthers from WT and *cdkg1-1* mutant plants illustrating normal tetrads in WT stage 7 anthers and aborted tetrads in the mutant (arrowheads). (Scale bar, 25 μ m.) (B) Chromatin configuration at different stages (as indicated to the Left) of meiosis in WT and mutant grown at 23 and 12 °C. Arrows indicate unsynapsed regions in a pachytene nucleus and univalents in metaphase I, and arrowheads indicate missegregated meiotic products. (Scale bar, 2 μ m.)

mammalian pachytene checkpoint (9), these data indicate that phosphorylation also modulates the degree of pairing in plants.

The origin and function of the Ph1-related CDKs is intriguing as they appear to be an orphan group, restricted to the monocotyledonous lineage. Based on BLAST against nonplant protein sequences and 3D modeling onto known protein structures, Ph1 kinases have been reported to resemble mammalian CDK2 in terms of predicted 3D structure (6). CDKG1 is the most similar plant kinase to Ph1 kinases in terms of primary sequence (10) and, consistent with this relationship, the data presented here indicate that CDKG1 acts on the same biological process, namely chromosome pairing. It will be interesting to undertake high-resolution characterization of the prophase defect in *ph1* mutant wheat when specific probes become available, but one might predict a similar defect in late zygotene/pachytene.

Bivalent formation relies on homologous recombination and synaptonemal complex polymerization, which physically binds homologs together via the establishment of COs (29–31). The normal localization of DMC1 and RAD51 proteins suggests that DSB formation and the early stages of repair are not affected by the loss of CDKG1. Elevated levels of DNA fragmentation were not observed in the mutant, implying that all DSBs are effectively repaired either via a non-CO recombination or via repair using the sister chromatid as a template. In addition, the lack of chromatin bridges at anaphase I indicates that DSBs were not repaired by nonhomologous end joining. In the absence of CDKG1, the level of class I crossovers, as detected by MutL homolog 1 (MLH1) staining, is dramatically reduced. The reduction of MLH1 foci in the mutant is likely a result of the reduced level of synapsis, as DSBs in unsynapsed regions lack the opportunity to engage in interhomolog recombination and are likely repaired via intersister recombination similar to what is observed during meiosis in haploid *Arabidopsis* (32).

Although the loading of the axis-associated protein ASY1 is not affected in the mutant, polymerization of the SC transverse filament protein ZYP1 is compromised, resulting in partial asynapsis. Links between chromosome structure and recombination have been proposed (29), and CDKG1 might act in this pathway. Another attractive possibility is that CDKG1 is part of a checkpoint mechanism that detects recombination defects and leads to meiotic arrest, as proposed for the RBR protein in *Arabidopsis* (5).

Temperature Stress, Splicing, and Male Meiosis. A possible alternative mechanism for CDKG1 action is less direct, via the regulation of gene expression. The large isoform of CDK11 (the mammalian homolog) promotes pre-mRNA splicing through its interaction with CyclinL (33), and a similar function has been proposed for CyclinL in plants (21), where it is required for the correct splicing of disease resistance transcripts. Indeed, CDKG1 was found to be associated with the spliceosome and regulates the splicing of a gene involved in pollen cell wall formation (34), indicating a general role in regulating several different aspects of pollen development.

The effect of temperature on meiosis and recombination frequency has long been recognized (35), and this study suggests a mechanism at least for male meiosis. In many species, pollen formation is particularly sensitive to stress, and plants are often sterile under conditions where vegetative growth can continue, leading to reductions in yield (reviewed by ref. 36). Moreover, male and female meiosis in *Arabidopsis* differ in terms of recombination rates (37). Reduction in CDKG activity reveals a pathway affecting the frequency of COs that is inherently temperature-sensitive and male-specific. Other work suggests that pairing and recombination is exquisitely sensitive to environmental conditions—a difference of 8 °C is sufficient to alter the balance of COs along the length of the chromosome in barley (38), and meiosis is more sensitive to temperature in polyploid wheat compared with its diploid relatives (39), but no mechanism was proposed in either case. The insertion mutation in CDKG1 leads to a loss of detectable transcript and could reveal the presence of a temperature-sensitive pathway. An alternative, and

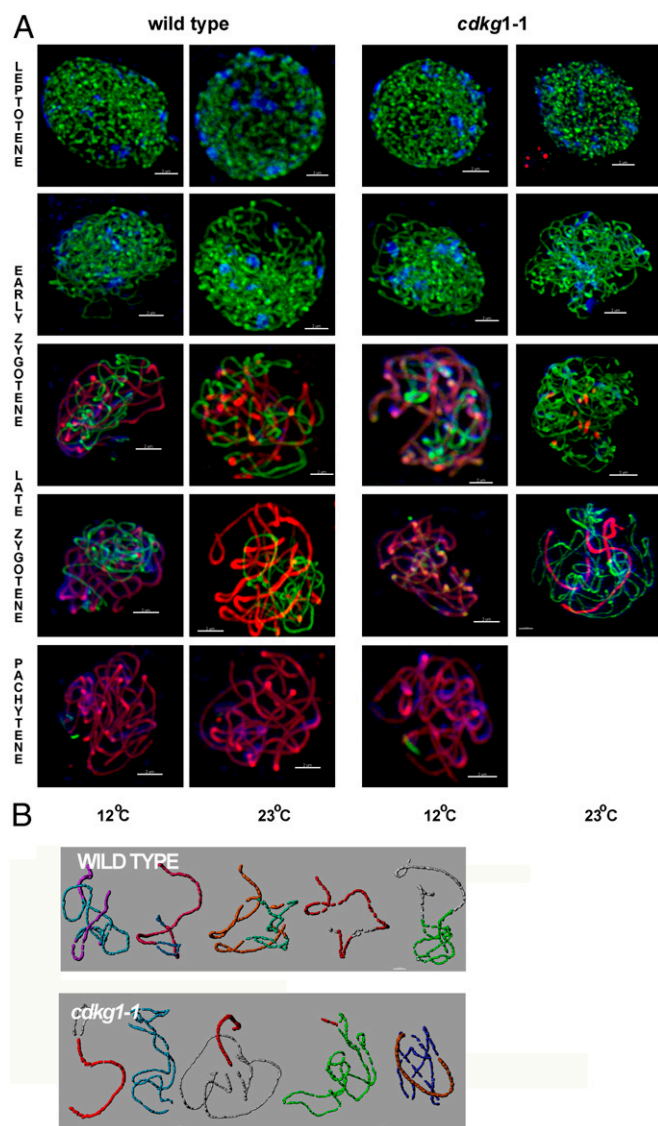


Fig. 3. Synaptonemal complex formation in WT and *cdkg1-1* mutant meiotic nuclei, isolated from plants grown at 12 or 23 °C. (A) Nuclei at different meiotic stages immunostained with ASY1 (green) and ZYP1 (red) with DAPI counterstain (blue). (Scale bar, 2 μ m.) (B) Three-dimensional reconstruction of individual bivalents from a pachytene WT nucleus (Upper) and pachytene-like mutant nucleus (Lower) at 23 °C. The nuclei were processed using Imaris, and each bivalent pair was isolated and false-colored. Quantification of ZYP1 loading is given in Fig. S3.

not mutually exclusive, mechanism would be that CDKG1 participates in a process that is itself thermodynamically unstable, and loss of CDKG1 enhances the inherent temperature sensitivity of the pathway. CDKG1 is evolutionarily conserved across both plants and animals and is implicated in mRNA processing (10), perhaps via regulation of alternative splicing (34). Alternative splicing often depends on the active selection of non-consensus splice sites that are thermodynamically unfavorable and have been suggested as a mechanism for allowing biological processes to adjust to ambient temperature variation (40, 41). Defining other targets of CDKG1-CYCLINL should provide further insight into the regulation of homologous chromosome pairing and holds promise for manipulating recombination in breeding, particularly of cereals, where the production of favorable recombinants can be rate-limiting.

Materials and Methods

The WT Columbia (Col-0) and mutant stocks [SALK_075762 (*cdkg1-1*); and SAIL_285_G10 (*cycl1-1*)] used in this study were obtained from the Nottingham *Arabidopsis* Stock Centre. Moist sterilized seeds were pretreated for 3 d at 4 °C in dark, then germinated on Petri-plates and transferred to soil after 1 wk or sown direct to soil. Plants were grown in a glasshouse (temperature range 14 to 25 °C) or in a growth chamber (16 h light; 60–70% relative humidity) at 23, 16, or 12 °C.

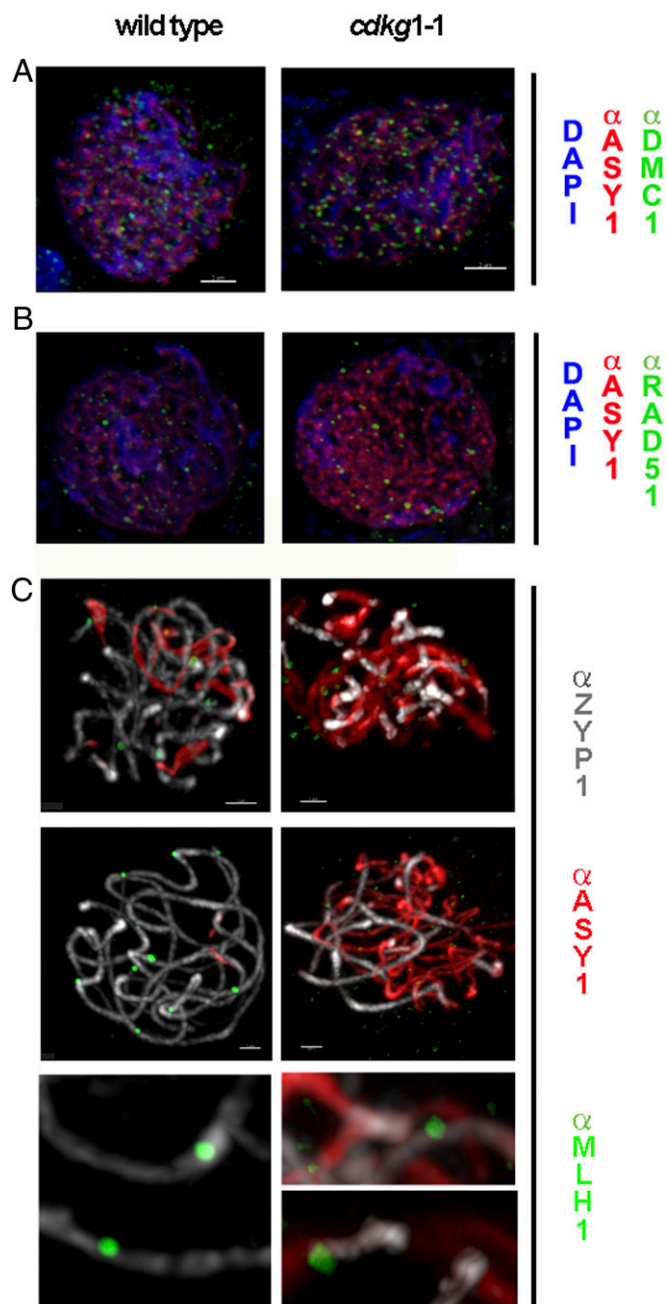


Fig. 4. Dual immunolocalization of SC proteins with recombination pathway proteins in WT and *cdkg1-1* nuclei at 23 °C. DAPI-stained chromatin is shown in blue. (Scale bars, 2 μ m.) (A) Leptotene nuclei immunostained with ASY1 (red) and DMC1 (green). (B) Leptotene nuclei immunostained with ASY1 (red) and RAD51 (green). (C) Late zygotene (Top) and pachytene or pachytene-like (Middle) nuclei of WT and *cdkg1-1* mutants at 23 °C. ASY1 (red), ZYP1 (gray), and MLH1 (green). (Bottom) Detail of MLH1 foci on ZYP1-loaded regions in both WT and mutant.

Genotyping and Cloning. Homozygous lines for the T-DNA insertion were identified by PCR using the LBA1 primer and a gene-specific primer (Table S2) and confirmed by sequencing. For the construction of the CDKG1 complementation plasmid, the CDKG1 promoter attB1 and Flg CDKG1 attB2 primers (Table S2) were used to amplify the entire CDKG1 coding sequence with flanking 5' and 3' UTR regions from genomic DNA. The PCR product was cloned into the binary vector pMDC123 by using the Gateway recombination system. The constructs were confirmed by DNA sequencing (carried out by Genome Centre of John Innes Centre).

RNA Extraction and RT-PCR. Tissue samples were collected and immediately frozen in liquid nitrogen. Tissues were ground in liquid nitrogen, and total RNA was isolated using the RNeasy mini kit (Qiagen). DNase treatment was carried out on 2 mg of total RNA using the DNase-free kit (Promega). One microgram of total RNA was reverse-transcribed using SuperScript III (Invitrogen) following the manufacturer's instructions using the primers indicated in Table S2.

Meiotic Spread Preparation. Whole inflorescences from *Arabidopsis* were fixed in 3:1 ethanol:acetic acid and stored at 4 °C. Flower buds in the size range 0.3–0.9 mm were used to prepare the squashes as described (42). Slides were then stained with DAPI for observation or used for FISH. FISH was used to identify individual bivalents, and chiasmata counts were recorded by light microscopy according to ref. 43.

Cytological Procedures. Mutant and WT pollen mother cells were examined by light microscopy in using FISH and DAPI stained spreads described by ref. 19. Pollen viability was assessed as described in ref. 44. Tissue samples were fixed in 2.5% (vol/vol) glutaraldehyde in 0.05 M sodium cacodylate, pH 7.3, vacuum-infiltrated, washed and dehydrated in an ethanol series (10, 20, 30,

50, 70, 95, and 100% ethanol) before infiltrating with and embedding in LR White resin (London Resin Company). For light microscopy, 0.5-μm-thick sections were dried onto glass slides and stained with 0.5% (wt/vol) Toluidine blue "O" in 0.5% (wt/vol) borax.

Immunolabeling *Arabidopsis* Pollen Mother Cells. Meicytes from WT and mutant plants growing at the different temperatures were embedded in acrylamide to preserve their 3D structure and used for the immunolocalization studies as described in ref. 22 with the following modifications. Buds of *Arabidopsis* plants in the prophase I meiotic stage (size range 0.3–0.9 mm) were isolated into Buffer A and fixed in 2% paraformaldehyde for 30 min. After 2 × 10-min washes in Buffer A the buds were macerated with a brass rod, and the suspension was then embedded in acrylamide. Immunolocalization using α-ASY1 (1:250), α-ZYP1 (1:200), α-DMC1 (1:200), α-RAD51 (1:200), and α-MLH1 (1:250) antibodies was performed, and images were acquired using a Leica TCS SP5II confocal microscope and Z-stacks deconvolved using AutoQuant ×2 (media Cybernetics). Bivalent tracking and analysis was performed using Imaris 7.3 (Bitplane).

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